Isolation, Screening and Characterization of Esterase Producing Bacteria from Oil Contaminated Soil

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Abstract:

The escalating release of industrial effluents and oil-laden wastes into the environment has emerged as significant ecological concern. These pollutants are characterized by substantial quantities of ester-based compounds, which are resistant to degradation through conventional treatment methodologies. Esterases, class of hydrolytic enzymes, facilitate the breakdown of short-chain esters. Environmental samples were collected from oil-contaminated sites from Bilimora, Gujarat. A total of 12 bacterial isolates (EB1–EB12) & 11 fungal isolates (EF1-EF11) were obtained. Based on primary screening for esterase activity, bacterial isolates EB6 and EB10 were selected for further study. Among these, isolate EB6 demonstrated the highest esterase-producing and was chosen for characterization. A total of esterase-producing isolates screened, with EB6 exhibiting the highest enzyme activity (12.75 ± 0.003U/ml) at 96 hours of fermentation. Consequently, EB6 was selected for further one-factor-at-a-time studies. The OFAT revealed that maximum enzyme production was supported by glucose (9.16 ± 0.003U/ml) as carbon source, yeast extract as nitrogen source (10.41 ± 0.003U/ml), sunflower oil as substrate (11.25 ± 0.003U/ml). Optimal physico-chemical conditions were identified at pH 7 and 3% inoculum size, resulting activities of (10.41 ± 0.003U/ml) and (11.66 ± 0.003U/ml) respectively.

1. INTRODUCTION:

Rapid industrialization and extensive petroleum-based activities have resulted in significant environmental contamination, particularly due to the accumulation of oil and hydrocarbon pollutants in soil ecosystems. Oil contamination mainly originates from petroleum refineries, industrial discharge, transportation leakage, and accidental oil spills, leading to the release of complex mixtures of hydrocarbons, fatty acids, triglycerides, and ester-containing compounds into the environment. These hydrophobic pollutants persist in soil for prolonged periods and negatively influence soil fertility, microbial ecology, and environmental sustainability (Altas et al., 2011).

Conventional physicochemical remediation techniques used for the treatment of oil-contaminated soils are often costly and may produce secondary pollutants. Therefore, biological remediation using microorganisms has gained considerable attention as an eco-friendly and sustainable alternative for environmental cleanup (Kim et al.,2010).

Enzymes are biological macromolecules synthesized by living organisms that act as highly efficient biocatalysts capable of accelerating biochemical reactions under mild environmental conditions without being consumed during the reaction process. Due to their remarkable

catalytic efficiency and substrate specificity, enzymes play essential roles in microbial metabolism, biodegradation, and nutrient cycling processes (Bairoch et al., 2000).

Among hydrolytic enzymes, esterases are important lipid-degrading enzymes that catalyse the hydrolysis of ester bonds present in short-chain fatty acid esters, resulting in the formation of alcohols and fatty acids (Bornscheuer et al., 2002). Esterases belong to the α/β-hydrolase enzyme family and possess a conserved catalytic triad responsible for efficient ester bond cleavage. These enzymes contribute significantly to the degradation of ester-containing organic pollutants present in oil-contaminated environments.

Oil-polluted soils represent favourable ecological niches for the isolation of esterase-producing microorganisms, as continuous exposure to hydrocarbons induces enzymatic adaptation and enhanced biodegradation capacity. The enzymatic hydrolysis of lipidic pollutants increases substrate bioavailability and facilitates microbial degradation of complex hydrocarbons, thereby contributing to environmental detoxification and restoration of contaminated ecosystem (Mussakhmetov et al., 2025).

2. MATERIALS &METHODOLOGY:

2.1 Collection of Samples:

For the present investigation, oil contaminated soil samples were collected from different locations of Bilimora. Samples were aseptically collected in sterile sampling container to avoid external contamination. The collected samples were immediately transported to the research laboratory under sterile conditions and stored at appropriate temperature for further microbiological analysis.

2.2 Isolation of Esterase Producing Bacteria:

Isolation of esterase-producing bacteria was carried out using the standard serial dilution technique. One gram of the collected soil sample was transferred into 9 ml of sterile distilled water to obtain a 10^{-1} dilution. Further serial dilutions were prepared up to 10-8. From each dilution, 0.1 ml aliquot was spread uniformly on Nutrient Agar plates under aseptic conditions. The plates were incubated at 30°C for 24 hours. After incubation, microbial colonies were observed and purified by repeated streaking. A total 21 of isolates were obtained from different samples (Alkabee et al., 2020).

2.3 Screening of Esterase Producing Bacteria:

The isolated bacterial strains were inoculated into 50 ml Nutrient Broth and incubated at 37 ºC. Screening for esterase production was performed using Tributyrin agar. The cup borer method was employed, where wells were prepared in the agar plates and inoculated with 0.1 ml of actively growing bacterial culture. Plates were incubated at room temperature for 24–48

hours. Formation of a clear hydrolysis zone around the wells indicated esterase enzyme production, and isolates showing larger hydrolysis zones were selected for further studies (Kumar et al., 2012).

2.4 Esterase Enzyme Assay:

Esterase activity was determined by selecting bacterial isolates exhibiting higher hydrolysis zones and inoculating them into esterase production medium. The inoculated cultures were incubated for 24 hours under shaking conditions as described by Winkler & Stuckman (1979). After incubation, 5 ml of culture broth was withdrawn and centrifuged at 6000 rpm for 20 minutes in a refrigerated centrifuge. The obtained supernatant was used as a source of extracellular esterase enzyme. Enzym activity was estimated using p-nitrophenyl acetate (p-NPA) as substrate. In the reaction mixture, 0.1 ml enzyme extract (supernatant) was mixed with 0.8 ml Tris–HCl buffer followed by addition of 0.1 ml p-NPA substrate solution and was kept in water bath at 60 ºC for 20 minutes. After incubation 0.25 ml of Na2Co3 was added to stop reaction. Further this reaction mixture was centrifuged at 11,000 rpm for 10 min. The release of p-nitrophenol was measured at 410 nm using a colorimeter, indicating esterase activity.

2.5 Identification of Bacterial isolate using morphological and Biochemical studies:

Selected esterase-producing isolates were characterized using standard biochemical tests. Pure cultures were maintained on nutrient agar.

2.6 Characterization of Bacterial Isolate for Esterase Enzyme Production:

The production of microbial esterases is strongly influenced by nutritional composition and environmental conditions such as carbon source, nitrogen source, substrate availability, pH, and inoculum size.

The potent esterase-producing Acinetobacter pittii was initially inoculated into 50 mL of production medium and incubated at 37°C under shaking conditions (120 rpm) for 24 h to obtain an actively growing inoculum. Activated culture was subsequently transferred into production medium containing supplemented with an appropriate substrate concentration to stimulate extracellular esterase synthesis. Similar optimization strategies have been widely employed for improving microbial esterase production (Kaiser et al.,2006).

2.6.1 Effect of Carbon Source on Esterase Production:

Carbon sources have a major impact on enzyme production and are crucial to microbial metabolism (Singh et al., 2016). Different carbohydrates, including as glucose, lactose, sucrose, and maltose, were added to the production medium separately to assess their impact on esterase synthesis (Acharya et al., 2016). A 24-hour-old isolate culture was added to the

medium, and it was then incubated at 37 °C. For five days, samples were taken every 24 hours, and a colorimeter was used to measure the esterase activity. Enzyme induction and microbial growth efficiency are known to be regulated by variations in carbon sources.

2.6.2 Effect of Nitrogen Source on Esterase Production:

Another important element affecting the synthesis of microbial enzymes is the availability of nitrogen **(Bharadwaj et al., 2020).** The production medium was supplemented with various organic nitrogen sources at a 1% (w/v) concentration, including **yeast extract, tryptone, urea, and peptone (Nadda et al., 2022).** A 24-hour-old culture was added to each flask, and it was then incubated under ideal circumstances. For up to seven days, esterase activity was evaluated using a colorimeter at 410 nm every 24 hours. By promoting cellular metabolism and protein synthesis, organic nitrogen sources have been shown to improve enzyme synthesis.

2.6.3 Effect of Substrate Type on Esterase Production:

Esterase enzyme production is largely regulated by substrate induction. A variety of natural lipid substrates, **such as sunflower, coconut, peanut, and olive oils**, were assessed as possible inducers of enzyme synthesis **(Acharya et al., 2016).** Isolates were added to production media that had been supplemented with the appropriate substrates, and the mixture was then incubated at 37 °C. After incubation, esterase activity was measured using a colorimeter at 410 nm. As enzymatic inducers, lipid substrates are known to increase the synthesis of esterase.

2.6.4 Effect of pH on Esterase Production:

Using suitable buffer systems, the production medium was adjusted to various pH levels **(5, 6, 7, and 8)** to investigate the impact of ambient pH on enzyme synthesis **(Nadda et al., 2022).** Enzyme activity was evaluated at regular intervals during the five days when the infected cultures were cultured at room temperature. Depending on extracellular pH levels that impact microbial growth and enzyme stability, microbial esterases typically display varied production patterns.

2.6.5 Effect of Inoculum size on Esterase Production:

The ideal conditions needed for maximal esterase production were identified by measuring the inoculum size. Production media that had been infected were incubated at inoculum sizes of **1%, 3%, 5%, and 7% (Bharadwaj *et al.*, 2020)**. Every 24 hours, samples were taken out, and the colorimeter at 410 nm was used to measure the enzyme activity. Microbial metabolic activity, catalytic efficiency, and enzyme production are all strongly impacted by inoculum size.

2.7 Partial Purification of Esterase enzyme:

Partial purification of the enzyme was carried out using fermentation broth obtained under both optimized and non-optimized conditions (Sayali et al., 2013). The crude extract was subjected to ammonium sulphate precipitation at 40% and 60% saturation levels with continuous gentle stirring for 2–3 hours to facilitate protein precipitation **(Alkabee *et al.*, 2020).** The mixture was then centrifuged at 12,000 rpm for 15 minutes to separate the precipitated proteins.

The protein pellets obtained were dissolved in a minimal volume of 0.25 mM Tris–HCl buffer (pH 7.0). To remove residual ammonium sulphate, dialysis was performed against the same buffer at 4°C using a suitable dialysis membrane. The buffer was replaced at regular 2-hour intervals to ensure efficient salt removal. Dialysis was continued until complete elimination of salts was achieved. The purified enzyme fractions were then analysed for total protein content and esterase activity, and the fraction exhibiting maximum activity was selected for further studies (**Hamid *et al.*, 2021).**

3. RESULT & DISCUSSION:

3.1 Collection of Samples:

Soil samples were collected aseptically from oil-contaminated sites, including automobile garages and food stall soil. These environments were chosen because constant exposure to lipid-rich waste helps microorganisms that can make lipolytic enzymes, especially esterases, grow.

3.2 Isolation of Esterase Producing Microorganisms:

Microorganisms were isolated from the collected soil samples using standard microbiological methods. Soil suspensions were serially diluted and spreading onto nutrient agar plates using the spread plate technique. After incubation, several morphologically distinct colonies were observed. A total 23 of isolates [bacterial (EB1-EB12) & fungal (EF1-EF11) were obtained from different samples.

3.3 Primary Screening of Esterase Producing Isolates:

Primary screening for esterase production was performed using tributyrin agar (TBA) plates. Individual colonies obtained from nutrient agar were inoculated onto TBA plates and incubated under suitable conditions. Tributyrin acts as a lipid substrate in the medium. Microorganisms capable of producing esterase enzymes hydrolyse tributyrin, resulting in the formation of clear zones of hydrolysis around the colonies Several of the bacterial isolates exhibited esterase enzyme activity. However, isolate **EB6(*Acinetobacter pittii*)** created the greatest hydrolysis zone **(34 mm)**, indicating the highest output of esterase among the examined bacterial strains.

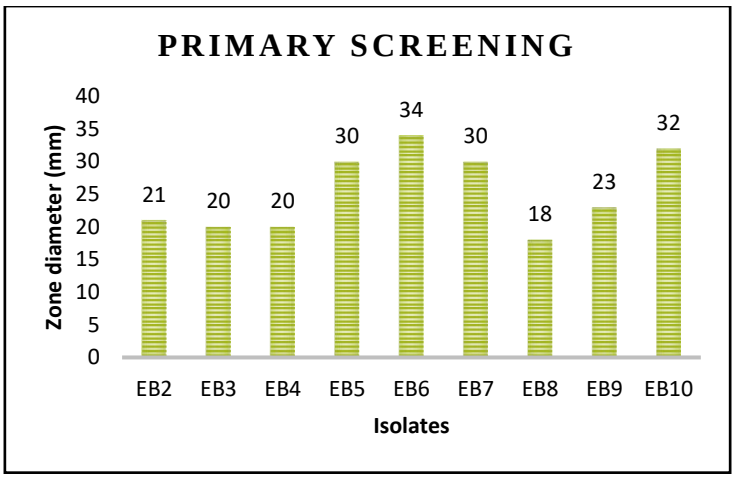

Figure 2: Primary Screening

3.4 Secondary Screening of Esterase Producing Isolates:

Secondary screening was carried out to quantitatively evaluate esterase activity in the culture broth of the isolates. Esterase activity was determined spectrophotometrically following the method described by **Winkler and Stuckmann (1979).** P-nitrophenol (p-NP) is released as a reaction product when p-nitrophenyl esters are hydrolysed, which is the basis of the assay. The substrate in this investigation was p-nitrophenyl acetate, or p-NPA. Using a colorimeter to detect the absorbance at 410 nm, the amount of p-nitrophenol emitted during the reaction was calculated.

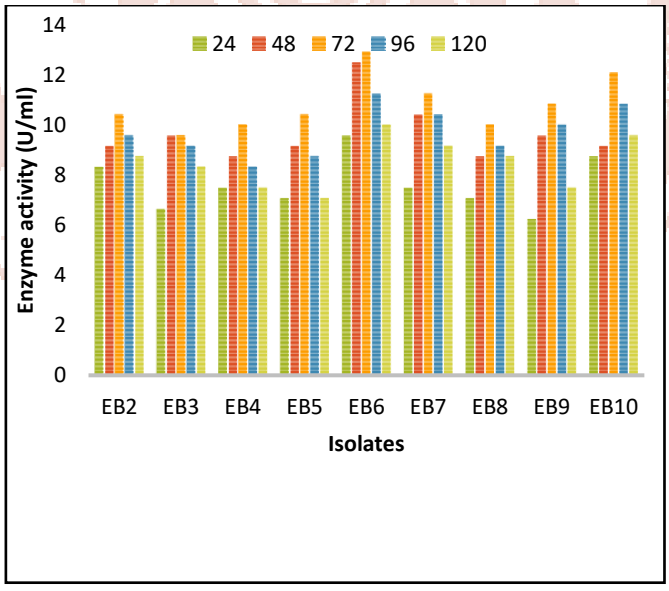

Figure3: Secondary Screening

Among all isolates, **EB6** showed the highest activity **12.41 ± 0.001 U/ ml** .The figure showed that the esterase activity of bacterial isolates at different incubation times. Enzyme activity increased up to 72 hours and then slightly decreased.

3.5 Identification of Esterase Producer:

A comprehensive morphological and biochemical study was performed on the chosen bacterial isolate, **EB6**, which showed the greatest esterase activity during primary screening. The isolate was rod-shaped and Gram-negative when examined under a microscope using Gram staining, indicating that it might belong to the genus

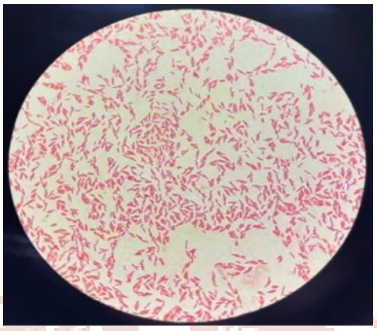

Figure 1: Gram Reaction

The isolate EB6 (code M6) was identified as ***Acinetobacter pittii***. Biochemical results shown in

Table 1: Biochemical Result of *Acinetobacter pittii*

Biochemical	Observation
Indole Test	-
Methyl Red Test	-
Voges -Proskauer Test	-
Simmon Citrate Test	+
Nutrient Gelatine Test	+
1% Peptone	-
2% Peptone	-
Triple Sugar Iron(TSI) Agar Test	Butt- Acidic
	Slant- Alkaline
	H_2S - -
	CO_2- +
Sugar:	
Glucose	AG
Lactose	AG
Maltose	AG
Manitol	AG
Sucrose	A

[This tables, (+) positive,(-) negative , AG indicates acid & gas , A indicates Acid]

3.6 Optimization of Esterase Production Using OFAT:

The One-Factor-At-a-Time (OFAT) method was used to optimize a number of physicochemical and nutritional factors to increase esterase production. This approach analysed each parameter's independent impact on esterase production by gradually changing

each one while maintaining the others constant. Various carbon sources, nitrogen sources, pH levels, substrates, and inoculum size were all the variables examined.

3.7 Characterization of Esterase Producing Acinetobacter Pittii:

3.7.1 Effect of Carbon source on Esterase Activity:

The selected isolate *Acinetobacter Pittii* demonstrated significant esterase-producing capability under submerged culture conditions. Among the tested substrates, glucose supported maximum enzyme production compared to other carbon sources. The highest esterase activity recorded with glucose was **9.16 U/ml.**

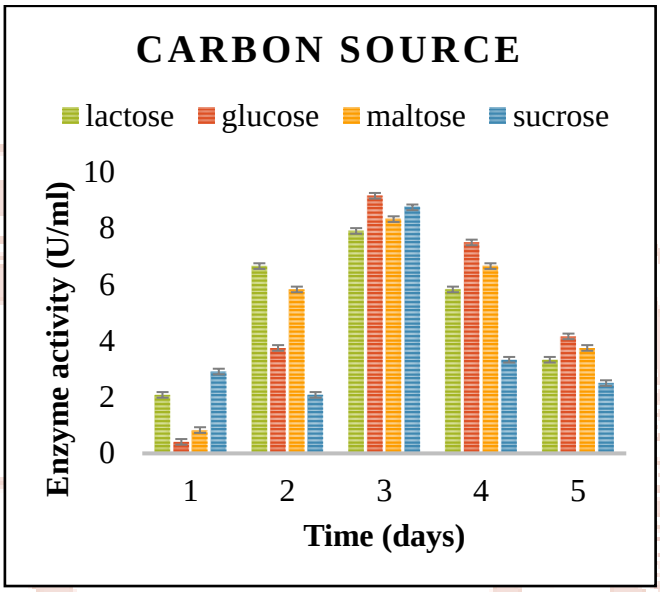

Figure 4: Effect of Carbon Sources

Several researchers have demonstrated that microbial isolates such as ***Rhodococcus, Bacillus, and Pseudomonas*** species show improved enzyme production when cultivated in glucose-supplemented media **(Kumar *et al.*,2012). Acharya *et al.*,2016**found that glucose was the best carbon source for *Bacillus subtilis KPA-1* esterase production, while **Bhardwaj *et al.*, (2020)** found that using galactose as the carbon source increased esterase activity in *Bacillus licheniformis*.

3.7.2 Effect of Nitrogen source on Esterase Activity:

Among the various nitrogen sources evaluated, isolate *Acinetobacter Pittii* exhibited maximum esterase enzyme activity when cultured in production medium supplemented with yeast extract, showing enhanced enzyme yield under optimized conditions. The highest esterase activity recorded with yeast extract was **10.41 U/ml**. Different nitrogen sources such as peptone, tryptone, yeast extract, and urea were examined.

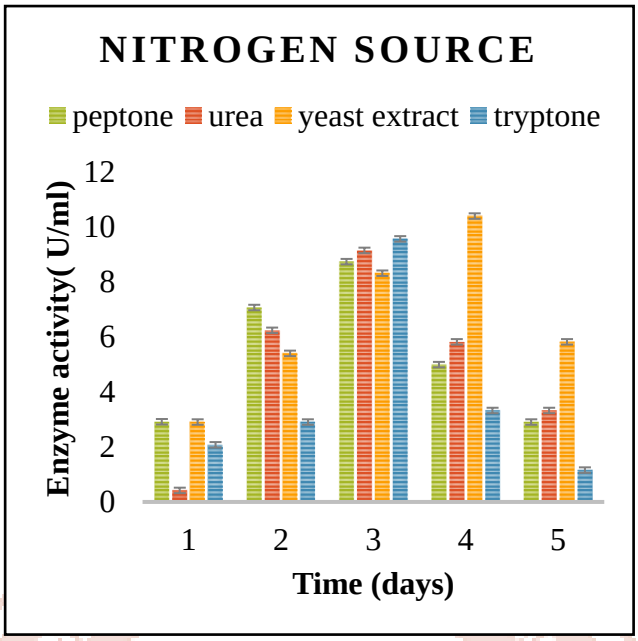

Figure 5: Effect of Nitrogen Sources

3.7.3 Effect of Substrate on Esterase Activity:

In the present study, the esterase-producing isolate *Acinetobacter Pittii* demonstrated maximum enzymatic activity when sunflower oil was used as a substrate compared to coconut oil, peanut oil, and olive oil, which resulted in comparatively lower enzyme production. The highest esterase activity recorded with sunflower oil was **11.25 U/ml**. The influence of different lipid substrates on enzyme activity was evaluated using substrate-based enzymatic assays similar to the Winkler and Stuckmann method.

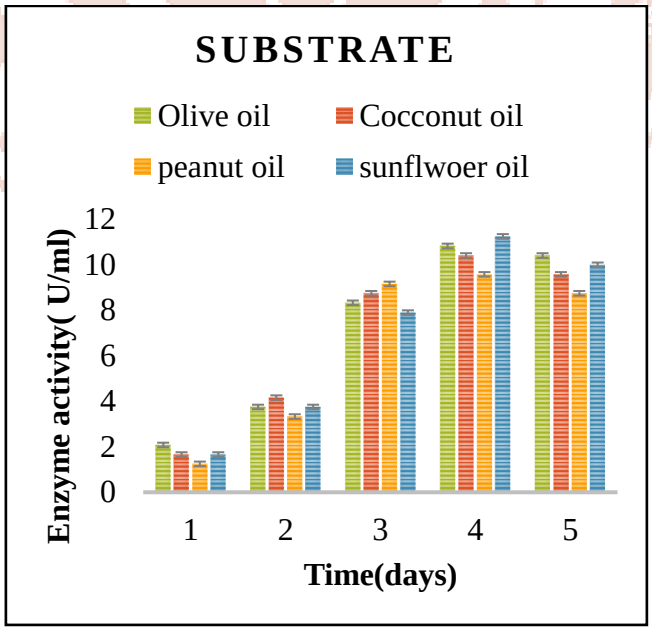

Figure 6: Effect of Substrates

Previous reports have demonstrated that Tween compounds stimulate esterase enzyme production in *Geobacillus and Pseudomonas* species **(Dahiya *et al.*,2022).** The findings of **Nadda *et al*. (2022)**, who also showed increased esterase synthesis in the presence of lipid-rich substrates, are in line with these results. In a similar to, **Bhardwaj *et al*. (2020)** found that using coconut oil as the substrate boosted the production of esterase in *Bacillus licheniformis*.

3.7.4 Effect of pH on Esterase Activity:

Optimization of environmental parameters revealed that isolate Acinetobacter Pittii exhibited maximum esterase activity at neutral pH (pH 7), showing enhanced enzyme production of **10.41 U/ml** compared to other tested pH conditions. pH plays a critical role in regulating enzyme stability, microbial growth, and catalytic efficiency. The optimization of pH is an important factor influencing microbial enzyme production, as it directly affects enzyme stability, catalytic efficiency, and microbial growth.

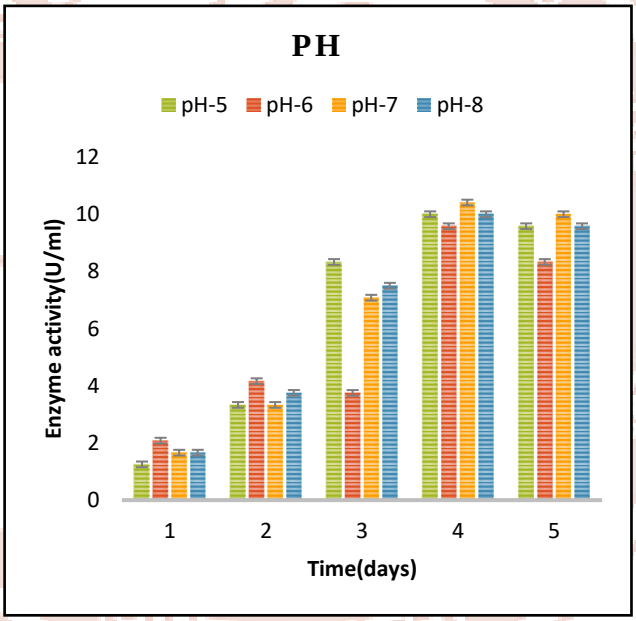

Figure 7: Effect of pH

Previous studies have reported that several esterase enzyme-producing bacteria exhibit alkalophilic characteristics **(Kim *et al.*, 2010).** These results align with earlier publications **Nadda *et al*. (2022)** found that the pH range of 6.5 to 9.5 was optimal for esterase production. In a similar vein**, Acharya *et al*. (2016)** found that *Bacillus subtilis KPA-1* produced the most esterase at pH 8.0, whilst **Bhardwaj *et al*. (2020)** found that *Bacillus licheniformis* exhibited maximal enzyme activity at pH 8.5. The majority of esterase-producing bacteria prefer neutral to slightly alkaline conditions for effective enzyme production.

3.7.5 Effect of Inoculum Size on Esterase Activity:

Inoculum size is another critical environmental parameter regulating enzyme production and metabolic activity. In the present study, esterase activity was evaluated across different inoculum sizes. The isolate *Acinetobacter Pittii* showed maximum enzyme production at 3%, recording an activity of **11.66 U/ml**. Generally, enzyme activity increases with rising inoculum size due to enhanced molecular collisions between enzyme and substrate molecules, which accelerates reaction rates.

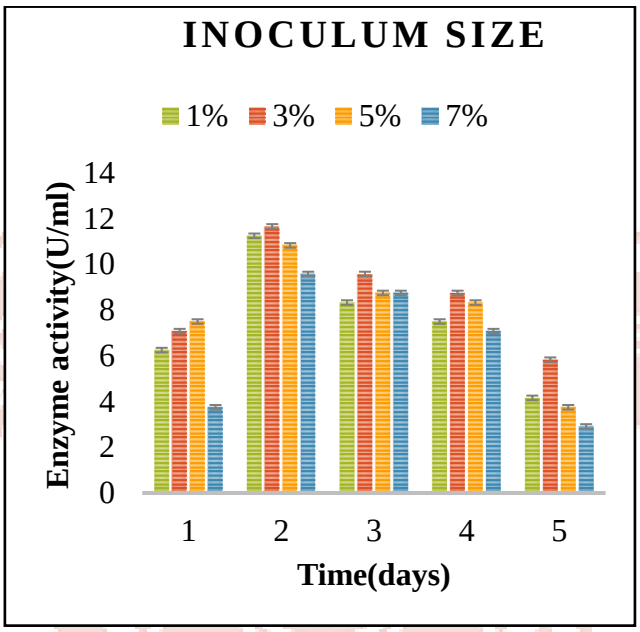

Figure 8: Effect of Inoculum Size

According to **Acharya *et al.* (2016)**, 1.5% is the ideal inoculum size for *Bacillus subtilis KPA-1* esterase production. On the other hand, 3% was shown to be a favourable inoculum size for Bacillus licheniformis by **Bhardwaj *et al.* (2020)**. These differences highlight how crucial it is to optimize inoculum size according to the particular microbial strain being employed.

3.8 Partial Purification of Esterase enzyme:

The crude enzyme extract was partially purified using ammonium sulphate precipitation at **40% and 60%** saturation levels. The 40% fraction showed moderate enzyme activity, while higher enzyme activity was observed in the 60% fraction, indicating better precipitation of the enzyme at this saturation level. The precipitated enzyme fraction was further purified by dialysis to remove excess ammonium sulphate.

Table 2: Partial Purification result of *Acinetobacter pittii*

Purification steps	Total enzyme activity (U/ml)	Total protein(mg/ml)	Specific activity	Fold purification
Crude	12.5	0.60	20.83	1
40% ASP	13.33	0.55	24.23	1.1
60% ASP	14.58	0.17	85.76	4.1
Dialysis	15.83	0.16	98.93	4.7

The fold purification obtained after dialysis was 4.7, demonstrating that the enzyme was significantly purified compared to the crude extract. This step also helped in stabilizing the enzyme preparation for further analysis **(Kumar et al., 2012;Sayali et al., 2013).**

4. CONCLUSION:

The study successfully isolated Acinetobacter pittii as a potent esterase producer with high enzyme activity. Partial purification confirmed the presence of active enzyme. This strain shows strong potential for industrial applications.

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